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14. ABSTRACT PAK6 is a serine threonine kinase whose expression is increased in prostate cancer. We are trying to understand the role played by PAK6 in PCa by finding its interacting partners. We have developed a strategy to find its interacting partners by tagging the protein with triple FLAG epitope, immunoprecipitating the protein using FLAG beads, elution by using triple FLAG peptide, running the eluted material on the gel, silver staining the gel and then mass spectrometry analysis is done on the specific bands. By this approach we have found nucleolin to be PAK6 interacting protein. Nucleolin is involved in cellular proliferation and it has also a role to play in cancer. We are in process of understanding the significance of this interaction in prostate cancer. Our another finding has shown that PAK6 is activated by MKK6 and p38 MAP kinase, so it implies PAK6 has some role to play under stress conditions. Nucleolin is also phosphorylated by p38 and its RNA binding ability increases under genotoxic stress. We are interested in understanding the link between these two independent findings.					
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INTRODUCTION:

PAK6 is a serine threonine kinase shown previously to interact with androgen receptor (AR) and ER α . PAK6 is expressed in normal prostate tissue but its expression is increased in prostate cancer. These initial studies support a role for PAK6 in PCa. In last one year we have tried to understand the role played by PAK6 in prostate cancer. We have developed a new strategy to understand the function of PAK6 by finding its interacting partners. We have also found a novel mechanism of activation of PAK6 by MKK6 and p38 MAP kinase and this finding is published and the published manuscript is attached.

BODY:

Task 1. Assess the effect of DHT stimulation in LAPC4 and LNCap cells expressing both AR and PAK6 endogenously on the

- a. Kinase activity of PAK6
- b. Interaction of AR and PAK6
- c. Colocalization of AR and PAK6

We are still working on this hypothesis as we are having problems with the immunoprecipitation of endogenous PAK6 using anti-PAK6 antibody. This antibody works very well for western blotting but we are not yet successful in using this antibody for immunoprecipitation. We had even tried very hard to use peptide antibodies for immunoprecipitation but could not get success.

To address this question we are trying to develop another strategy. We have developed stable LNCap cells expressing triple FLAG PAK6 and in this cell line immunoprecipitation using FLAG antibody works very well. We might be doing our experiments using this approach although PAK6 is transfected in this cell line and not endogenously present but AR is present endogenously.

Task 2. Assess the role of PAK6 in enhancing tumorigenicity by determining the effects of blocking PAK6 in PCa cell lines by siRNA. The role of PAK6 in enhancing tumorigenicity will also be determined by overexpressing PAK6 in vitro and in vivo

We tried to block PAK6 in PCa cell lines by siRNA but we failed in this attempt. We had used a pool of four siRNA oligos to knock out PAK6 in breast cancer cell lines but the same pool is not working for PCa cell lines. The reason might be the transfection efficiency is very less in these cell lines.

We have developed tet regulated PAK6 expressing clones but the problem with these clones is they are leaky. They express PAK6 prior to doxycycline induction. We are still in the process of developing clones which express PAK6 only after doxycycline induction and no PAK6 in the absence of doxycycline induction.

The main aim of Task 2 is to assess the function of PAK6. Although we tried to knock out PAK6 by RNAi approach and also made an effort to develop a strategy to overexpress PAK6 but we were not quite successful in that.

We have developed another approach to understand function played by PAK6 in prostate cancer. This approach involves finding the interacting partners of PAK6. The proteins with which PAK6 is interacting physiologically will say a lot about function of PAK6. By this approach we might be able to figure out in which pathway PAK6 falls and what might be its function.

We have developed three stable cell lines expressing triple FLAG PAK6 in LanCaps, MCF7 and 293 T cells. From these cell lines immunoprecipitation using FLAG beads is very efficient as triple FLAG is expressed on the surface of protein and is accessible to the FLAG monoclonal antibody covalently bound to the beads. Triple FLAG PAK6 along with its associated proteins bound to beads is then eluted using triple FLAG peptide. The eluate from cells expressing PAK6 along with control cell line not expressing PAK6 is run on the gel and then this gel is stained by silver staining. The bands other than the PAK6 band which are specific for the cell line expressing PAK6 are cut with razor blade and sent for mass spectrometry. By this approach we have found nucleolin, 100kDa nucleolar protein involved in the ribosome biosynthesis to be the interacting partner of PAK6 in 293 T cells. We are in the process of understanding the role of this interaction in this cell line as well as in MCF7 and LanCaps as nucleolin is expressed in MCF7 and LanCaps endogenously. We also hope to find another partners of PAK6 in these stable cell lines using the same strategy as it worked very well.

Apart from the objectives stated in the proposal we have also worked on the activation of kinase activity of PAK6 and successfully found that PAK6 is activated by MAP kinase kinase 6 and p38 MAP kinase. We have published a manuscript from this finding in JBC which is attached with this report and the details of the experiments can be seen in the manuscript. In short the findings of the manuscript are as follows. The p21-activated kinases (PAKs) contain an N-terminal Cdc42/Rac interactive binding domain, which in the group 1 PAKs (PAK1, 2, and 3) regulates the activity of an adjacent conserved autoinhibitory domain. In contrast, the group 2 PAKs (PAK4, 5, and 6) lack this autoinhibitory domain and are not activated by Cdc42/Rac binding, and the mechanisms that regulate their kinase activity have been unclear. Our study found that basal PAK6 kinase activity was repressed by a p38 mitogen-activated protein (MAP) kinase antagonist and could be strongly stimulated by constitutively active MAP kinase kinase 6 (MKK6), an upstream activator of p38 MAP kinases. Mutation of a consensus p38 MAP kinase target site at serine 165 decreased PAK6 kinase activity. Moreover, PAK6 was directly activated by MKK6, and mutation of tyrosine 566 in a consensus MKK6 site (threonine-proline-tyrosine, TPY) in the activation loop of the PAK6 kinase domain prevented activation by MKK6. PAK6 activation by MKK6 was also blocked by mutation of an autophosphorylated serine (serine 560) in the PAK6 activation loop, indicating that phosphorylation of this site is necessary for MKK6-mediated activation. PAK4 and PAK5 were similarly activated by MKK6, consistent with a conserved TPY motif in their activation domains. The activation of PAK6 by both p38 MAP kinase and MKK6 suggests that PAK6 plays a role in the cellular response to stress-related signals. The manuscript is attached with the report.

KEY RESEARCH ACCOMPLISHMENTS:

- Published Manuscript (see Appendices)

REPORTABLE OUTCOMES:

- 293 T cells stably expressing triple FLAG PAK6
- MCF7 stably expressing triple FLAG PAK6
- LanCap stably expressing triple FLAG PAK6

CONCLUSIONS:

PAK6 is a kinase which interacts with AR and ER and also its expression is increased in prostate cancer. In our last one year study we have tried to understand the function of PAK6 in prostate cancer. Although the approaches to address this question we proposed in our proposal have not worked

.We are still in the process of working on them and we have also developed new strategies to address the same question.To understand the role played by PAK6 in prostate cancer we have developed stable cell lines expressing triple FLAG PAK6 and we have used FLAG beads for immunoprecipitation and they work very well .PAK6 immunoprecipitated on FLAG beads with its interacting partners is eluted by using FLAG peptide and the eluted material is run on the gel and silver staining is done and the specific bands are cut and sent for mass spectrometry.By this strategy we have found nucleolin to be the interacting partner of PAK6 and this protein is involved in cellular growth and it's expression also increases in cancer(1).We are in process of understanding the significance of this interaction in prostate cancer.We have also found PAK6 is activated by MKK6 and p38 MAP kinases which suggests PAK6 has some role to play under stress conditions.Nucleolin's RNA binding ability is also increased under genotoxic stress and it is also mediated by p38 MAP kinase(2) .So we are in process of understanding the connection of these two independent findings.

REFERENCES:

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2. C Yang *et al.*,*Nucleic Acids Res.***30**,2251(2002)

APPENDICES:

See the published Manuscript.

Activation of p21-activated Kinase 6 by MAP Kinase Kinase 6 and p38 MAP Kinase*

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The p21-activated kinases (PAKs) contain an N-terminal Cdc42/Rac interactive binding domain, which in the group 1 PAKs (PAK1, 2, and 3) regulates the activity of an adjacent conserved autoinhibitory domain. In contrast, the group 2 PAKs (PAK4, 5, and 6) lack this autoinhibitory domain and are not activated by Cdc42/Rac binding, and the mechanisms that regulate their kinase activity have been unclear. This study found that basal PAK6 kinase activity was repressed by a p38 mitogen-activated protein (MAP) kinase antagonist and could be strongly stimulated by constitutively active MAP kinase kinase 6 (MKK6), an upstream activator of p38 MAP kinases. Mutation of a consensus p38 MAP kinase target site at serine 165 decreased PAK6 kinase activity. Moreover, PAK6 was directly activated by MKK6, and mutation of tyrosine 566 in a consensus MKK6 site (threonine-proline-tyrosine, TPY) in the activation loop of the PAK6 kinase domain prevented activation by MKK6. PAK6 activation by MKK6 was also blocked by mutation of an autophosphorylated serine (serine 560) in the PAK6 activation loop, indicating that phosphorylation of this site is necessary for MKK6-mediated activation. PAK4 and PAK5 were similarly activated by MKK6, consistent with a conserved TPY motif in their activation domains. The activation of PAK6 by both p38 MAP kinase and MKK6 suggests that PAK6 plays a role in the cellular response to stress-related signals.

p21-activated kinases (PAKs)¹ were originally identified as serine/threonine protein kinases that bound to and were activated by the p21 GTPases, GTP-Cdc42 and -Rac. Binding of p21 is mediated by an N-terminal Cdc42/Rac interactive binding (CRIB) domain, and biochemical and crystal structure analyses of PAK1 have shown that the CRIB domain regulates the inhibitory activity of an adjacent autoinhibitory domain (AID).

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¹ The abbreviations used are: PAK, p21-activated kinase; MKK6, MAP kinase kinase 6; CRIB, Cdc42/Rac interactive binding domain; AID, autoinhibitory domain; MKP-1, mitogen kinase phosphatase-1; WT, wild type; HA, hemagglutinin; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; IP, immunoprecipitation; mAb, monoclonal antibody.

In the absence of p21 GTPase binding, PAK1 exists as an autoinhibited dimer in which the N-terminal AID of one PAK1 molecule in the dimer binds to the other catalytic domain and blocks its function. Binding of GTP-Cdc42 or -Rac causes the AID to dissociate from the catalytic domain and activates its kinase activity, with subsequent phosphorylation of sites in the N-terminal regulatory domain and in the activation loop of the kinase domain serving to maintain the activated state (1, 2).

The N-terminal CRIB domain and AID are highly conserved in human PAK2 and PAK3, and these PAKs have been categorized with PAK1 as group 1 PAKs. PAK6 was initially identified in yeast two-hybrid screens for androgen receptor-interacting proteins (3, 4). PAK6 has a C-terminal kinase domain with homology to the group 1 PAKs and an N-terminal CRIB domain. However, PAK6 lacks the conserved AID and is not stimulated by ligation of its CRIB domain, which binds selectively to GTP-Cdc42 (3). Human PAK4 and PAK5 similarly lack the conserved AID and along with PAK6 comprise the group 2 PAKs (5).

Group 1 PAKs (PAK1, PAK2, and PAK3) are involved in the regulation of diverse cellular processes such as cell motility, morphology, cytoskeletal reorganization, and gene regulation. Much less is known about the regulation and function of group 2 PAKs (PAK4, PAK5, and PAK6). PAK4 is expressed ubiquitously, and activated PAK4 has been shown to mediate cytoskeleton reorganization and filopodia formation (6, 7). Targeted disruption of PAK4 results in embryonic lethality. PAK5 is highly expressed in brain and neuronal tissues and has been shown to promote neuron outgrowth during development. RNA blot analyses have shown that PAK6 is expressed most highly in brain and testes and at lower levels in multiple tissues including prostate and breast. In transfection studies, PAK6 has been shown to suppress androgen receptor transcriptional activity and similarly bind to and repress estrogen receptor (3).

The mechanisms that regulate the group 2 PAKs are unclear, but the absence of a conserved AID indicates that the modes of regulation differ from the group 1 PAKs. In the current study, we describe a novel mechanism of PAK6 regulation by the MKK6-p38 MAP kinase pathway. Our results demonstrate that MKK6 activates PAK6 by targeting two separate sites, a consensus p38 MAP kinase substrate site (Ser-165) and a tyrosine (Tyr-566) in the activation loop of the kinase domain. Significantly, this tyrosine is part of an MKK6 substrate motif (threonine-X-tyrosine) that is conserved in the group 1 and 2 PAKs but is otherwise largely restricted to activation loops of MAP kinases, where it undergoes direct dual phosphorylation by MAP kinase kinases. This study further shows that MKK6-mediated activation does not alter the autophosphorylation of a regulatory serine in the activation loop of PAK6 (Ser-560), which is also conserved in the activation loop of all PAKs.

Moreover, this serine is required for MKK6-p38 MAP kinase activation of PAK6. Taken together, the results in this study indicate that PAK6 is regulated by MKK6 and p38 MAP kinase and that the PAK6 activation loop is regulated by both MKK6 and autophosphorylation.

EXPERIMENTAL PROCEDURES

Materials and Reagent—p38 MAP kinase inhibitor SB203580 and MEK1 inhibitor PD98059 were purchased from LC Laboratories (Woburn, MA). cAMP-dependent protein kinase activator forskolin and histone H4 were purchased from Sigma. c-Jun NH₂-terminal kinase inhibitor SP600125, PI3K inhibitor LY 294002, and MEK1 inhibitor U0126 were purchased from Calbiochem. Protein-A-conjugated Sepharose beads were from Amersham Biosciences. Monoclonal antibodies against phosphotyrosine and p38 MAP kinase were from Upstate Biotechnology (Lake Placid, NY), and monoclonal antibody 12CA5 against the hemagglutinin (HA) tag was from Berkeley Antibody (Berkeley, CA). Antiserum against phospho-PAK4(Ser-474)/PAK5(Ser-602)/PAK6(Ser-560) was purchased from Cell Signaling Technology (Burlington, MA). The QuikChange mutagenesis kit was from Stratagene Inc. (La Jolla, CA). Anti-PAK6 polyclonal antiserum was generated against glutathione S-transferase fused with PAK6 residues 115–383.

Expression Vectors and Constructs—PAK6 was cloned in-frame with an N-terminal HA-tag containing pcDNA3.0 vector (Invitrogen). N-terminal tagged MKK6(EE) was a gift from Dr. R. Davis (8). Generation of S165A and Y566F point mutations were done by PCR-based site-directed mutagenesis QuikChange kit following the manufacturer's instructions. The mutation primers used for S165A are: CCG TGG CCC GAG CCA CAG GAA CCA CGG GTC CTG CCC AAT GGG and CCG TGG CCC GAG CCA CAG GAA CCA CGG GTC CTG CCC AAT GGG. The primers used for Y566F are: TCC CTG GTG GGA ACC CCC TTC TGG ATG GCT CCT GAA GTG and CAC TTC AGG AGC CAT CCA GAA GGG GGT TCC CAC CAG GGA. The primers used for S560A (S560D or S560E) are: GAC GTC CCT AAG AGG AAG GCC (or GAC (for D) or GAA (for E)) CTG GTG GGA ACC CCC TAC and GTA GGG GGT TCC CAC CAG TTC (or GTC (for D) or TTC (for E)) CTT CCT CTT AGG GAC GTC. The site-directed mutagenesis was performed based on the manufacturer's protocol.

Cell Culture and Transient Transfection—HEK293 cells were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with antibiotics and 10% fetal bovine serum. The cells were transfected by electroporation with a total of 10 μ g of plasmid DNA using a Gene Pulser from Bio-Rad.

Gel Electrophoresis and Immunoblotting—The proteins were separated by SDS-PAGE with a standard reducing protocol. Following electrophoresis, the proteins were electroblotted to a nitrocellulose membrane. The protein bands were visualized by Ponceau S red staining. The blots were blocked by 5% nonfat dry milk, 0.05% Tween 20, and 1% bovine serum albumin in Tris-buffered saline (10 mM Tris, pH 8.0, 135 mM NaCl). Immunoblotting was performed with designated antibodies and visualized with an ECL detection system (Pierce) following the manufacturer's protocol.

Immunoprecipitation—Immunoprecipitation of PAK6 and proteins containing phosphotyrosine was employed following a standard protocol. In brief, the cells were lysed in immunoprecipitation radioimmune precipitation assay buffer containing 50 mM Tris, pH 7.4, 135 mM NaCl, 1% (v/v) Triton X-100, 0.25% (w/v) deoxycholate, and 0.05% (w/v) SDS and supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 5 mM diisopropyl fluorophosphate, 5 μ g/ml pepstatin, 1 mM EDTA). The lysates were cleared by centrifugation at 12,000 $\times g$ for 30 min at 4 $^{\circ}$ C. The supernatants were incubated with individual antibodies (1 μ g) and protein A-Sepharose beads (20 μ l of packed beads) at 4 $^{\circ}$ C for 1 h. At the end of incubation, the beads were washed five times with lysis buffer. The resulting immunoprecipitated immunocomplexes were solubilized in 40 μ l of Laemmli sample buffer, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. The protein complex was detected by Western blot analysis and developed by ECL (Pierce; Supersignal).

In Vitro Kinase Assay—Kinase reactions of immunoprecipitated PAK6 were performed in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl₂, 2 mM MnCl₂, and 2 mM dithiothreitol, 200 μ M ATP) supplemented with 2.5 μ g/reaction of histone H4 and 20 μ Ci/reaction of radioactive ATP. The reactions were incubated for 30 min at 30 $^{\circ}$ C and stopped by the addition of sample buffer containing SDS. The reactions were resolved by SDS-PAGE, and autoradiography of radiolabeled protein was performed.

In Vitro Kinase Assay:

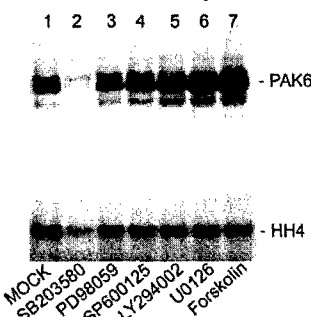


FIG. 1. Inhibition of PAK6 kinase activity by p38 MAP kinase inhibitor SB203580. Various agents were used to treat HEK293 cells transiently transfected with HA-PAK6 for 24 h. The cells were treated with pharmacological agents for 1 h before being subjected to the immunoprecipitation protocol. PAK6 was immunoprecipitated from cell lysates with anti-HA mAb 12CA5, and its kinase activity was assayed in the presence of histone H4 and [γ -³²P]ATP. Both autophosphorylation and substrate phosphorylation were analyzed after SDS-PAGE and autoradiography. The upper panel shows the autophosphorylation of PAK6. The lower panel shows the phosphorylation of exogenously added histone H4 substrate. The dosages used were: SB203580, 25 μ M; PD98059, 100 μ M; SP600125, 10 μ M; LY294002, 20 μ M; U0126, 50 μ M; and forskolin, 10 μ M.

RESULTS

PAK6 Is Inhibited by p38 MAP Kinase Antagonist—In contrast to PAK1, PAK6 exhibits readily detectable basal kinase activity even in the absence of exogenous stimulation. To determine the molecular mechanisms that regulate PAK6 kinase activity, we tested a group of agents with known specificity either as inhibitors or as activators of their respective pathways. HEK293 cells were transiently transfected with HA-tagged PAK6 for 24 h and then treated with drugs for 1 h prior to immunoprecipitation with an anti-HA antibody. Kinase activities in the immunoprecipitates were then measured by *in vitro* kinase assays, using histone H4 as an exogenous substrate. Among nine tested agents, only the p38 MAP kinase inhibitor SB203580 exhibited inhibitory effects on PAK6 kinase activity, with reduced autophosphorylation and reduced phosphorylation of the exogenous histone H4 substrate (Fig. 1).

PAK6 Is Activated by p38 MAP Kinase Upstream Activator MKK6—Inhibition of PAK6 kinase activity by SB203580 suggested that the p38 MAP kinase pathway was regulating PAK6 activity. To further test this possibility, we co-transfected HEK293 cells with HA-tagged PAK6 and a constitutively active MKK6, MKK6(EE), an upstream activator of p38 MAP kinase (9, 10). The effect on PAK6 kinase activity was then assessed by *in vitro* immunoprecipitation kinase assays. As shown in Fig. 2A, MKK6(EE) caused an increase in PAK6 autophosphorylation and histone H4 phosphorylation. Immunoblotting of the immunoprecipitates with the anti-HA antibody confirmed that PAK6 protein expression was not altered, indicating that MKK6(EE) increased PAK6 kinase activity (Fig. 2B).

The involvement of p38 MAP kinase in this PAK6 activation by MKK6(EE) was examined by assessing the inhibitory effect of SB203580. Significantly, although SB203580 markedly down-regulated PAK6 activity in the absence of MKK6(EE), it only partially inhibited the MKK6(EE)-induced activation of PAK6 (Fig. 2C). This partial inhibition was consistent with the high level of p38 MAP kinase activation in the MKK6(EE)-transfected cells (Fig. 2D). However, the substantial PAK6 activation in the MKK6(EE)-transfected and SB203580-treated cells also suggested a p38 MAP kinase-independent mechanism for PAK6 activation.

To further address the role of p38 MAP kinase in PAK6 activation, we attempted to identify a site that was phospho-

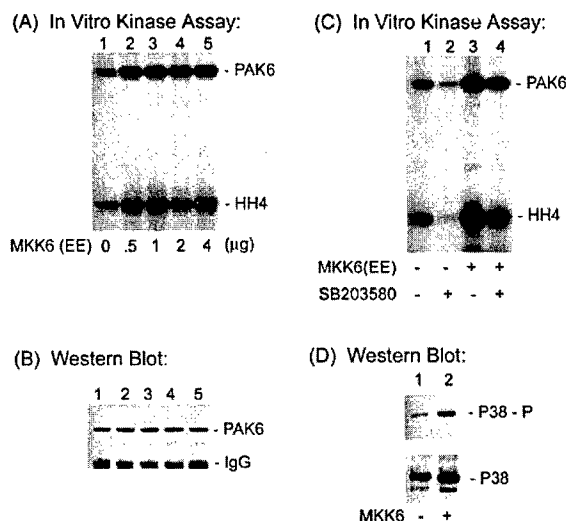


FIG. 2. MKK6-induced PAK6 activation is down-regulated by SB203580. A, 293 cells were transiently co-transfected with both HA-PAK6 and various dosages of constitutively active mutant MKK6(EE). *In vitro* kinase activity was assessed by a kinase assay on the immunoprecipitated PAK6. B, equal amounts of PAK6 from the immunoprecipitation in A were immunoblotted with anti-HA. C, the effect of SB203580 on MKK6-activated PAK6 kinase activity was measured by an IP/kinase assay using cell lysates of 293 cells transiently co-transfected with MKK6 and PAK6 that were treated with or without SB203580. D, the phosphorylation status of p38 MAP kinase in response to MKK6 activation was confirmed by Western blot analysis using an anti-p38 phospho-specific antibody (upper panel) or total p38 (lower panel).

regulated by p38 MAP kinase. Aided by an on-line kinase substrate site analysis, Scansite (scansite.mit.edu), we identified a potential p38 MAP kinase phosphorylation site at serine 165 of PAK6. To characterize the role of Ser-165 in p38 MAP kinase-mediated PAK6 activation, we generated a serine-to-alanine (S165A) substitution mutant of PAK6 by site-directed mutagenesis. Consistent with SB203580 down-regulation of PAK6 kinase activity, substitution of Ser-165 with alanine dramatically reduced basal PAK6 kinase activity (Fig. 3A, lane 1 versus lane 3) as well as MKK6-stimulated activity (Fig. 3A, lane 2 versus lane 7). However, despite the down-regulation of kinase activity in the PAK6 S165A mutant, it remained responsive to MKK6-induced activation in a dose-dependent manner (Fig. 3A). Fig. 3B shows that the serine-to-alanine mutation, or MKK6 co-transfection, did not markedly alter PAK6 protein expression. Taken together, these findings indicated PAK6 could be activated by p38 MAP kinase-mediated phosphorylation of serine 165 but also suggested a second mechanism for activation by MKK6.

PAK6 Activity in Response to MKK6 Is Regulated by Sites in the Kinase Domain—The result that the S165A PAK6 mutant remained responsive to MKK6 indicated that additional target site(s) in PAK6 might be involved in p38 MAP kinase-mediated activation. To test this hypothesis, a series of PAK6 deletion mutants were employed to map additional region(s) of PAK6 that may participate in the MKK6-p38 MAP kinase-induced activation. Although the basal activities varied, co-transfected MKK6(EE) remained effective in up-regulating the kinase activities of PAK6 deletion mutants that extend from the N terminus to the region covering only the catalytic domain (Δ368 deletion mutant) (Fig. 4A). The levels of expression of wild type and deletion mutants in the control and MKK6(EE) co-transfected groups were similar, as shown in Fig. 4B. This result suggested that additional sites in the kinase domain were susceptible to activation by MKK6.

PAK6 Is Phosphorylated on Tyrosine upon MKK6 Activation—To identify potential MKK6-p38 MAP kinase target res-

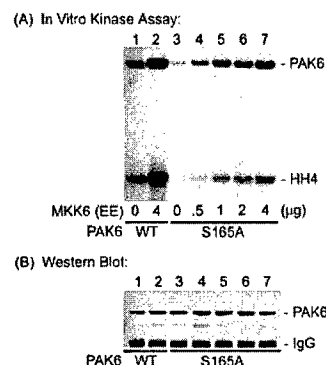


FIG. 3. Substituting serine 165 with alanine down-regulates MKK6-induced PAK6 activation. A, 293 cells were transiently co-transfected with HA-PAK6 WT or S165A and with various dosages of constitutively active mutant MKK6(EE). *In vitro* kinase activities were determined by a kinase assay on the immunoprecipitated PAK6. B, anti-HA Western blot of PAK6 WT and S165A mutants in A.

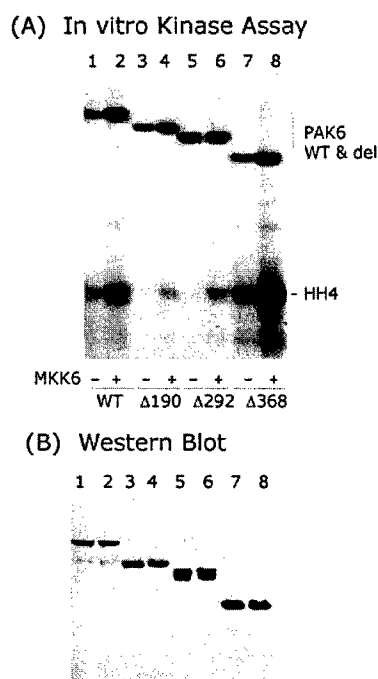


FIG. 4. Mapping PAK6 domain susceptible to MKK6-induced activation. A, 293 cells were co-transfected with MKK6(EE) and HA-tagged PAK6 WT full length and N-terminal deletion mutants Δ190, Δ292, or Δ368, respectively, for 24 h (all with N-terminal HA tags). The kinase activities of PAK6 WT, and N-terminal deletion mutants were determined by an IP kinase assay using anti-HA 12CA5 mAb. B, Western blot analysis showed equal levels of expression of PAK6 full length and deletions.

idue in the PAK6 kinase domain, we examined the sequence of PAK6 within this domain. No additional candidate p38 MAP kinase sites were found, but the activation loop contained a threonine-proline-tyrosine (TPY) sequence (residues 564–566) that resembled the substrate motif (TXY) recognized by MKK6 (14, 15). This motif was also present in the activation loop of MAP kinases and PAK1–6 but not in most other kinases (see Fig. 8A). MKK6 is a dual specificity kinase that recognizes and phosphorylates both threonine and tyrosine residues on the TXY motif of its substrate. The identification of TPY within the PAK6 activation loop suggested that PAK6 might be a direct substrate of MKK6. If this is the case, then one should detect increased tyrosine phosphorylation of PAK6 upon MKK6(EE)-induced activation. Reciprocal immunoprecipitation (IP)/Western blot analyses were performed to test this possibility.

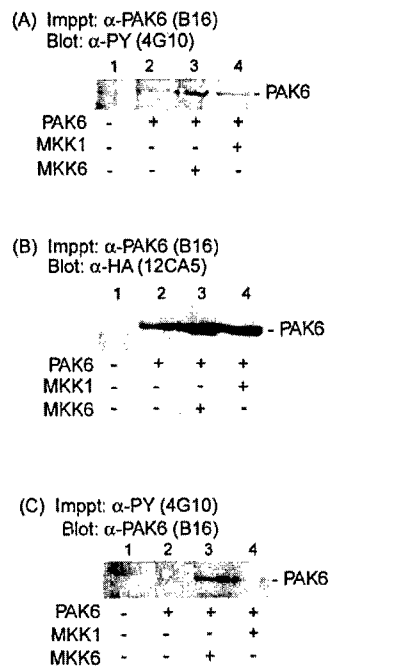


FIG. 5. Tyrosine phosphorylation of PAK6 by MKK6 but not by MKK1. 293 cells were co-transfected with HA-PAK6 and MKK6(EE) or MKK1(DD) and further incubated for 24 h. PAK6 was immunoprecipitated with anti-PAK6(B16) antiserum or with anti-phosphotyrosine (α -PY) 4G10 mAb. The immunoprecipitates were resolved by SDS-PAGE followed by reciprocal Western blot analysis. In both A and C, an increased tyrosine phosphorylation of PAK6 was evident in MKK6(EE) co-transfected group. B, comparable levels of PAK6 in the anti-PAK6(B16) immunoprecipitates were demonstrated by Western blot using anti-HA mAb.

PAK6 was immunoprecipitated from transfected cells with a polyclonal antibody raised against PAK6 (B16) and then immunoblotted with anti-phosphotyrosine antibody (4G10). An increase in tyrosine phosphorylation in the anti-PAK6 immunoprecipitates was detected when PAK6 was co-expressed with MKK6(EE) (Fig. 5A). In contrast, no change in tyrosine phosphorylation of PAK6 was detected when it was co-transfected with the constitutively active MKK1(DD), a related member of the MAP kinase kinase family (16). Immunoblotting with anti-HA showed that the immunoprecipitates contained comparable levels of total PAK6 (Fig. 5B). In the reciprocal experiment, the lysates were immunoprecipitated with the anti-phosphotyrosine 4G10 antibody and then immunoblotted for PAK6. As shown in Fig. 5C, MKK6 co-transfection increased the level of PAK6 that was immunoprecipitated by anti-phosphotyrosine 4G10.

To further address the phosphorylation of Tyr-566 by MKK6, a dual specificity phosphatase, MKP-1, which can dephosphorylate both threonine and tyrosine on the TXY motif (17), was tested in IP/Western blot experiments. MKP-1 co-transfection in the absence of MKK6(EE) markedly decreased the basal tyrosine phosphorylation of PAK6 (Fig. 6, A and B, lane 1 versus lane 3). MKP-1 co-transfection similarly reduced the level of MKK6-induced tyrosine phosphorylation of PAK6 (Fig. 6, A and B, lane 2 versus lane 4). Fig. 6C demonstrates that total PAK6 expression levels were not altered by MKP-1 (lane 1 versus lane 3 and lane 2 versus lane 4). Finally, *in vitro* kinase assays were carried out to assess the correlation between the loss of tyrosine phosphorylation induced by MKP-1 and PAK6 kinase activity. Consistent with the tyrosine phosphorylation results, MKP-1 down-regulated basal and MKK6-stimulated PAK6 kinase activity on the exogenous histone H4 substrate (Fig. 6D). Basal autophosphorylation was also decreased by

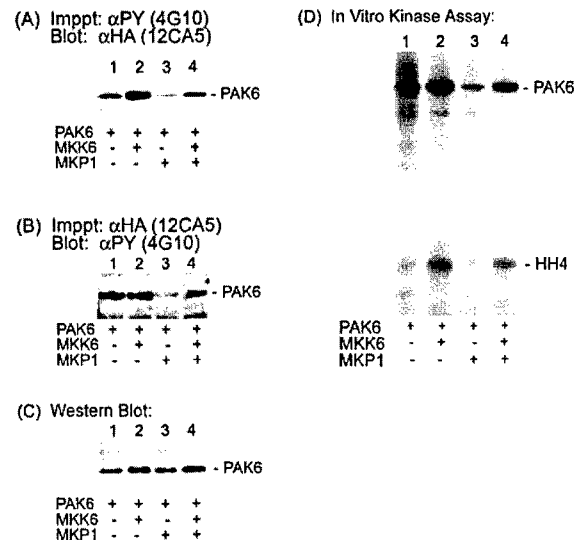


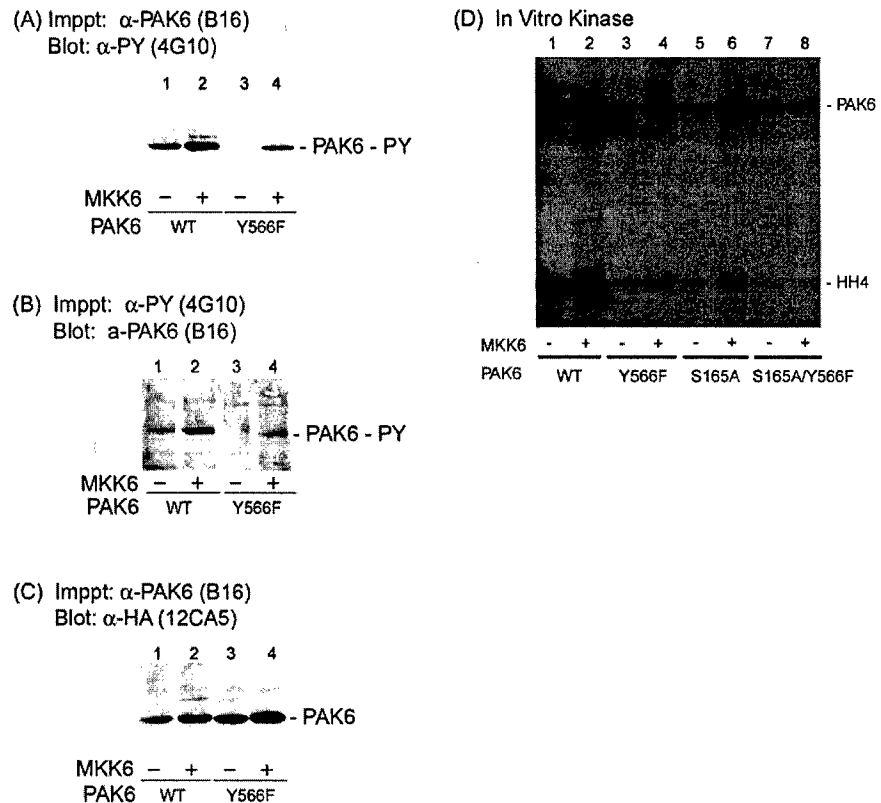
FIG. 6. Down-regulation of MKK6-induced PAK6 tyrosine phosphorylation and kinase activation by dual specificity phosphatase MKP-1. 293 cells were co-transfected with HA-PAK6 and MKK6(EE) or MKP-1 and further incubated for 24 h. PAK6 was immunoprecipitated with anti-HA 12CA5 mAb or with anti-phosphotyrosine (α -PY) 4G10 mAb. The immunoprecipitates were resolved by SDS-PAGE followed by reciprocal Western blot analysis. The results are shown in A and B. MKP-1 dephosphorylates PAK6 tyrosine residue under basal and MKK6-stimulated conditions, as evident from the reduced immunoreactivity of PAK6 with anti-phosphotyrosine 4G10 mAb. C, comparable amounts of PAK6 in lysates derived from each group were demonstrated by Western blot analysis. D, down-regulation of PAK6 kinase activity by MKP-1 co-transfection was demonstrated by an *in vitro* kinase assay using 293 cells co-transfected with PAK6 and with MKK6(EE) or MKP-1. Kinase activity was determined by an *in vitro* kinase assay with [32 P]ATP using the anti-HA immunoprecipitated kinase and analyzed by SDS-PAGE followed by autoradiography.

MKP-1, although the effects on autophosphorylation in the MKK6-transfected cells were less prominent.

Substitution of Tyr-566 with Phenylalanine Down-regulates PAK6 Activation by MKK6—To assess more directly the involvement of Tyr-566 phosphorylation in PAK6 activation, we generated a mutant PAK6 (Y566F) by substituting the Tyr-566 residue with a phenylalanine. This mutation markedly reduced the level of basal PAK6 tyrosine phosphorylation (Fig. 7, A and B, lane 1 versus lane 3). Similarly, the Y566F mutation markedly reduced the tyrosine phosphorylation stimulated by MKK6 (Fig. 7, A and B, lane 2 versus lane 4). Fig. 7C demonstrates that the wild type and mutant PAK6 constructs were expressed at comparable levels. These results indicated that Tyr-566 was a major site of basal and MKK6-stimulated tyrosine phosphorylation, although perhaps not the only site, because the Y566F mutation did not completely eliminate MKK6-induced tyrosine phosphorylation.

We next examined the effects of the Y566F mutation on basal and MKK6-stimulated PAK6 kinase activity. Substitution of Tyr-566 with phenylalanine reduced basal PAK6 autophosphorylation and kinase activity toward the exogenous histone H4 substrate (Fig. 7D, lane 1 versus lane 3) and reduced the magnitude of MKK6-stimulated PAK6 kinase activation (Fig. 7D, lane 2 versus lane 4). Moreover, a double mutation of S165A and Y566F completely abrogated the MKK6-stimulated PAK6 activation (Fig. 7D, lane 8). Therefore, although MKK6 may directly or indirectly stimulate the phosphorylation of additional tyrosines, serine 165 and tyrosine 566 appear to be the critical sites mediating MKK6-stimulated PAK6 kinase activity. Taken together, these data indicate that MKK6 activates PAK6 by direct phosphorylation of the TXY motif located

FIG. 7. Substitution of Tyr-566 with phenylalanine (Y566F) dampens MKK6-stimulated PAK6 activation. Levels of tyrosine phosphorylation were evaluated between PAK6 WT and Y566F mutant by IP/Western blot analysis using 293 cells transiently expressing MKK6(EE) and PAK6 (WT or Y566F). PAK6 was immunoprecipitated with anti-HA 12CA5 mAb or with anti-phosphotyrosine (α -PY) 4G10 mAb. The immunoprecipitates were resolved by SDS-PAGE followed by reciprocal Western blot. The results are shown in A and B. Reduced levels of tyrosine phosphorylation of PAK6 in basal and MKK6-stimulated conditions were evident in the Y566F group. C, Western blot demonstrated the comparable amount of PAK6 in anti-HA immunoprecipitates. D, *in vitro* IP Kinase assay demonstrated down-regulation of PAK6 kinase activity by substituting tyrosine 566 with phenylalanine (Y566F) at both basal nonstimulated and MKK6-stimulated conditions (lanes 3 and 4). Double mutation of serine 165 and tyrosine 566 (S165A/Y566F) abrogated MKK6-induced PAK6 activation (lanes 7 and 8).



within the activation loop and by stimulating p38 MAP kinase-mediated phosphorylation of serine 165.

MKK6/p38 MAP Kinase Pathway Regulates Other Members of PAK Family—As indicated above, the activation segments of PAK1–6 all contain similarly positioned TPY sequences (Fig. 8A). These are located four amino acids C-terminal to conserved serines (PAK4–6) or threonines (PAK1–3), which are autophosphorylated and also regulate kinase activity (see below). This TXY motif is absent from most other serine/threonine kinases, and the positioning of the motif in the MAP kinases is distinct. These observations suggest that activation induced by MKK6 may be common among PAK family kinases. Therefore, additional members of the PAK family (HA-tagged PAK1, PAK4, and PAK5) were tested in the same co-transfection studies coupled with *in vitro* kinase assays.

Consistent with previous reports, PAK4 and PAK5 had readily detectable basal kinase activities. Both PAK4 (Fig. 8B, lanes 5 and 6) and PAK5 (Fig. 8B, lanes 3 and 4) responded to MKK6(EE) co-transfection with, respectively, 4.7- and 3.2-fold increases of kinase activity in a fashion similar to PAK6 (Fig. 8D, lower panel). In contrast, PAK1 was inactive both in the absence and presence of MKK6(EE) (Fig. 8, B, lanes 7 and 8, and D, lower panel) but was strongly stimulated by co-transfection with a constitutively active valine 12 mutant of Cdc42 (Cdc42-V12). However, MKK6(EE) co-transfection had a marginal effect on further promoting Cdc42-activated PAK1 activity, with no change in enzymatic activity toward exogenous substrate histone H4 and a small 22% increase of autophosphorylation (Fig. 8, B, lanes 9 and 10, and D). Immunoblotting with an anti-HA antibody confirmed that each of the kinases was expressed at comparable levels within each group (Fig. 8C). These results indicate that members of the group 2 PAK family (PAK4, 5, and 6) share a common mechanism of being stimulated via the MKK6-p38 MAP kinase pathway.

Serine 560 Phosphorylation in Activation Loop Is Required for MKK6-mediated Stimulation—A serine residue conserved

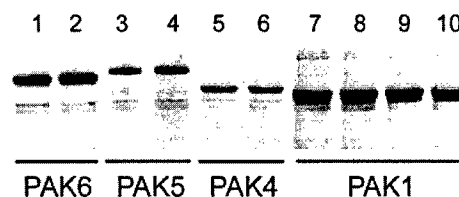
among group 2 PAKs is located at position 560 within the activation loop of the PAK6 kinase domain (Fig. 8A). This serine residue corresponds to the autophosphorylated regulatory threonine 423 in the activation loop of PAK1 and was previously shown (using a phosphoserine 560-specific antibody) to be autophosphorylated under basal conditions in PAK6 (11). This phospho-specific antibody was used to determine whether MKK6-p38 MAP kinase activation increased phosphorylation at this site. As shown in Fig. 9A, MKK6 co-transfection did not alter Ser-560 phosphorylation level (lane 1 versus lane 2). The specificity of the antibody was confirmed by the lack of reactivity to mutants in codon 560 (lanes 3–5), and equivalent total PAK6 expression was confirmed by immunoblotting for the HA epitope tag (Fig. 9B). Finally, although MKK6 did not increase Ser-560 phosphorylation, we next determined whether phosphorylation at this site was necessary for PAK6 activation by MKK6-p38 MAP kinase. Significantly, mutation of this site to alanine (S560A) markedly diminished the basal activity and abrogated stimulation by MKK6 (Fig. 9C, lanes 3 and 4).

In PAK1, substitution of threonine 423 with a glutamic acid (T423E) that mimics a negatively charged phosphothreonine residue results in constitutive activation of the kinase, whereas substituting threonine with alanine ablates kinase activity (12, 13). To further characterize the role of serine 560 phosphorylation in PAK6 activation, we generated PAK6 mutants by substituting the serine 560 with either negatively charged glutamic acid (S560E) or aspartic acid (S560D). The enzymatic activities of these mutants were tested along with wild type PAK6 and MKK6-p38 MAP kinase activated wild type PAK6. As shown in Fig. 9C (lanes 5 and 7), substitution of negatively charged residues (S560E or S560D) did not stimulate kinase activity but instead markedly repressed basal kinase activity. Similarly to the S560A mutation, these mutations also completely abrogated PAK6 activation by MKK6 (lanes 6 and 8). Taken together, these results indicated a critical MKK6-inde-

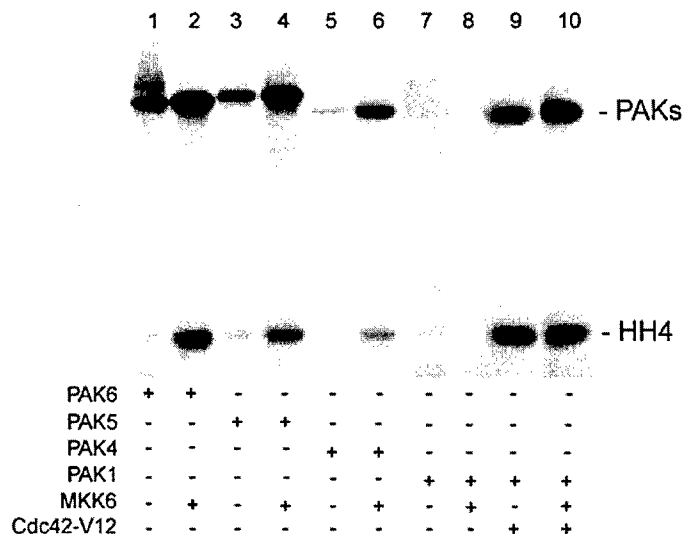
(A) Kinase Activation Domain Alignment:

KINASE	Subdomain	
	VII	VIII
PAK6	DFGFCQAISKDVPRK---SLVG	TPYWMAPE
PAK5	DFGFCQAQVSKEVPRK---SLVG	TPYWMAPE
PAK4	DFGFCQAQVSKEVPRK---SLVG	TPYWMAPE
PAK1	DFGFCQAQITPEQSKRS---TMVG	TPYWMAPE
PAK2	DFGFCQAQITPEQSKRS---TMVG	TPYWMAPE
PAK3	DFGFCQAQITPEQSKRS---TMVG	TPYWMAPE
p38a	DFGLARHTDDEM-----TGY	VATRWRYPE
p38b	DFGLARQADEEM-----TGY	VATRWRYPE
JNK1	DFGLARTAGTSFMM-----TPY	VVTRYRYPE
JNK2	DFGLARTACTNFMM-----TPY	VVTRYRYPE
Erk1	DFGLARIADPEHDHTGFL	TEYVATRWRYPE
Erk2	DFGLARVADPDHDHTGFL	TEYVATRWRYPE

(C) Western Blot:



(B) In Vitro Kinase Assay:



(D) Quantitation of Kinase Reaction:

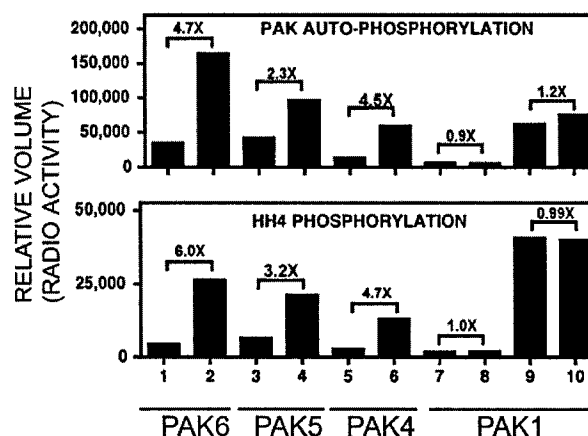


FIG. 8. Specificity of MKK6-induced PAK activation. A, sequence alignment of the activation loop region between catalytic subdomains VII and VIII of PAKs and MAP kinase family members. In both group 1 and group 2 PAKs, in addition to the previously defined autophosphorylated threonine or serine residues, a conserved TXY motif was also found within the activation loop. B, kinase activity of MKK6-modulated PAKs. 293 cells were co-transfected with MKK6(EE) and one of the following HA-tagged PAK family members: PAK1, PAK4, PAK5, or PAK6. Additionally, PAK1 was also tested by co-transfecting with a constitutive active Cdc42-V12. The kinase activity was determined by an *in vitro* kinase assay with anti-HA mAb immunoprecipitated kinases and analyzed by SDS-PAGE followed by autoradiography. C, Western blot demonstrated the comparable level of expression within individual PAK testing group. D, quantitation of kinase reactions from B using a PhosphorImager. The upper panel depicts levels of autophosphorylation of various PAKs. The lower panel depicts kinase activity toward histone H4. The numbers on top of each PAK group indicate fold activation in response to MKK6 co-transfection.

pendent role for serine 560 autophosphorylation in regulating PAK6 kinase activity.

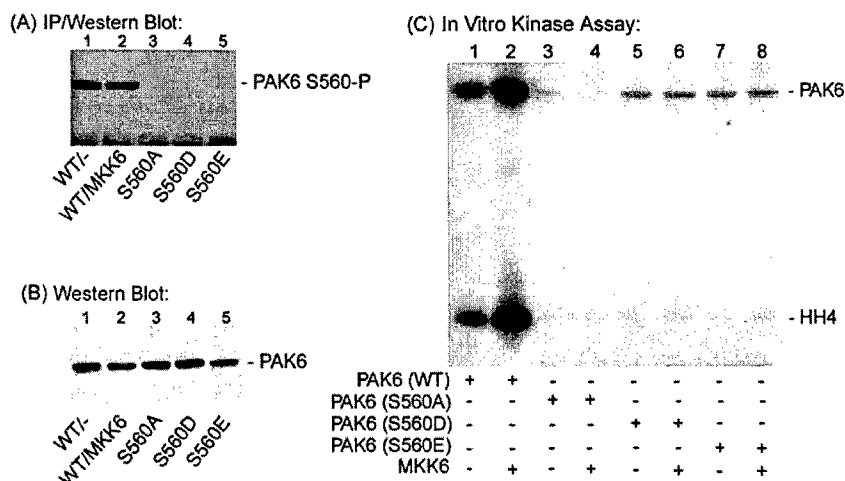
DISCUSSION

PAK6 is classified as a PAK family member based on homology in the kinase domain and in its N-terminal CRIB domain. However, in contrast to PAK1 and the other group 1 PAKs, PAK6 kinase activity is not stimulated by Cdc42 or Rac binding, and the mechanisms that regulate its kinase activity have been unclear. This study found that basal PAK6 kinase activity was repressed by a p38 MAP kinase antagonist and could be strongly stimulated by activation of the MKK6-p38 MAP kinase pathway. A role for p38 MAP kinase in directly regulating PAK6 was further supported by a marked decrease in kinase

activity upon mutation of a consensus target site at serine 165. PAK6 was also directly activated by MKK6, and this activation was dependent upon tyrosine 566 in the activation loop of the PAK6 kinase domain. Significantly, this tyrosine is part of an MKK6 dual specificity kinase substrate motif, TXY, that is found in the activation loop of MAP kinases and in the other PAKs but is absent from most other kinases. Finally, PAK6 kinase activity was also dependent upon an autophosphorylated serine (serine 560) in the activation loop. These results indicate that PAK6 kinase activity is regulated by both autophosphorylation and MAP kinase kinase-mediated phosphorylation of residues in its activation loop and by p38 MAP kinase mediated phosphorylation outside the kinase domain.

The kinase activity of PAK1 is regulated by an AID in the

FIG. 9. PAK6 does not respond to MKK6-induced activation with increased Ser-560 phosphorylation. A, 293 cells transiently co-transfected with HA-tagged PAK6 Ser-560 substitution mutants, serine to alanine (S560A), serine to aspartic acid (S560D), or serine to glutamic acid (S560E), were immunoprecipitated by anti-HA. 12CA5 monoclonal antibody was subjected to Western blot with an anti-Ser-560 phospho-specific antibody. B, anti-HA Western blot showed comparable amount of PAK6 protein expressed in the each testing group. C, PAK6 Ser-560 substitution mutants S560A, S560D, and S560E along with wild type were transfected without or with MKK6(EE), and the resulting kinase activity was analyzed by IP/kinase assays.



N-terminal half of the molecule downstream of the CRIB domain, which binds to and represses the catalytic domain (1, 2, 12, 18–21). The binding of GTP-Rac or GTP-Cdc42 to the CRIB domain causes the AID to dissociate from the catalytic domain, with subsequent phosphorylation of residues in the AID and of Thr-423 in the activation loop of the catalytic domain (12, 13, 18, 22). In contrast to PAK1 and the other group 1 PAKs (PAK2 and PAK3), this AID is not conserved in PAK6 or in the other group 2 PAKs (PAK4 and PAK5), and the kinase activities of the group 2 PAKs are not stimulated by Cdc42 binding. Nonetheless, the group 2 PAKs contain serine at a position homologous to the autophosphorylated threonine 423 of PAK1 (serine 560 in PAK6). Significantly, constitutive autophosphorylation of this serine 560 in PAK6 was recently demonstrated using a phospho-specific anti-PAK6-Ser-560 antibody (11), consistent with the lack of an AID.

Thr-423 in PAK1 and Ser-560 in PAK6 are located at the center of the activation loop within the catalytic domain. Crystallographic studies of PAK1 indicate that the phosphorylation of this residue stabilizes the interaction between the activation loop and substrate (1). Consequently, substituting Thr-423 with a negatively charged glutamic acid residue renders PAK1 constitutively active. In PAK4, mutating the corresponding Ser-473 to glutamic acid also results in constitutive kinase activity (23, 24). In contrast, we found that substituting the PAK6 Ser-560 with glutamic acid or aspartic acid diminished kinase activity, indicating that these negatively charged residues were not equivalent to phosphorylation in the case of PAK6. More importantly, replacement of serine 560 with alanine resulted in the almost complete abrogation of kinase activity, confirming a critical role for this site.

Although PAK6 does not appear to have an AID, and Ser-560 phosphorylation appears to be constitutive and required for kinase activity, we cannot yet rule out the possibility that Ser-560 phosphorylation also negatively regulates PAK6 interaction with an undefined inhibitory domain. One recent report describes as the "autoinhibitory" domain of the PAK5 a region that bears almost no sequence homology to other members of the PAK family (25). This region (residues 60–180) is located directly downstream of the PAK5 CRIB domain and appears to be able to down-regulate PAK5 kinase activity *in vitro*. This result raises the interesting possibility that regions immediately following the CRIB domain of group 2 family PAKs may regulate the catalytic domain. If this is the case for PAK6, then the identification of serine 165 as a p38 MAP kinase substrate site would suggest that PAK6 activation may be initiated by phosphorylation of this site and that this activation signal may

then be amplified by Ser-560 phosphorylation and subsequent MKK6 phosphorylation of the activation loop.

The highly conserved activation loops in PAK1–6, each containing the TXY motif, indicate that dual regulation by autophosphorylation and MKK6 may be common to other members of the PAK family. Indeed, the kinase activities of PAK4 and PAK5 were also stimulated by MKK6, consistent with the MKK6 regulation of the group 2 PAKs. In contrast, in response to MKK6 co-transfection, only a marginal increase of Cdc42-activated PAK1 autophosphorylation was observed, but its kinase activity toward exogenous substrate histone H4 remained unchanged. This was not due to a dominant inhibitory effect of the AID, because MKK6 failed to enhance the activation mediated by activated Cdc42. These results suggest that activation by MKK6 may be unique among the group 2 PAKs, although it is possible that there are more subtle effects on PAK1 or that additional priming events are needed. Alternatively, the TPY motif in the group 1 PAKs may be recognized by another kinase.

Although the PAK and MAP kinase families appear to share a functional TXY MAP kinase kinase motif, its position is shifted toward the C terminus of the activation loop in the PAKs. Moreover, PAKs differ from the MAP kinases in that they have a regulatory serine or threonine located in the center of the activation loop. Further structural studies are needed to determine precisely how phosphorylation at these multiple sites affects the activation loop and kinase activity. Nonetheless, the shared mechanism of activation by PAK6 and p38 MAP kinase suggests that PAK6 (and likely other PAKs) has a unique and specialized role in the cellular response to stress-related signals.

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